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(54) Title: ESTIMATION OF ACTIVE INFECTION BY HELICOBACTER PYLORI

(57) Abstract

Disclosed is a diagnostic apparatus for estimating an active Helicobacter pylori infectious agent in saliva, comprising in combination an immunoassay chamber in which a first portion of said saliva is subjected to chemical analysis for an ammonia constituent thereof.

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ESTIMATION OF ACTIVE INFECTION BY HELICOBACTER PYLORI

Technical Field

This invention relates generally to methods and devices used for determination of analytes in saliva or other body fluid, and in particular, pertains to an integrated device and method to carry out chemical and immunochemical analysis simultaneously and including an improved device for determination of ammonia.

Background Art

Helicobacter pylori (formerly called Campylobacter pylori) was first isolated by Warnell and Shall in 1983 (Marshall B.J., Warren J. R., Lancet 1984:1:1311-5). H. pylori is the most widespread bacteria infection with an estimated worldwide prevalence of 50% (Marshall B.J., Epidemiology of H. pylori in Western countries. In: Hunt R.H., Tytgat G.N. J., eds. Helicobacter pylori: Basic Mechanisms to Clinical Cure. Dordrecht:; Kluwer Academic Publishers 1994:75-84; Hazell S. .., H. pylori in developing countries. In: Hunt R.H., Tytgat G.N. J., eds. Helicobacter pylori: Basic Mechanisms to Clinical Cure. Dordrecht:; Kluwer Academic Publishers 1994:85-94). H. pylori is a very important pathogen in several diseases of the stomach and duodenum. H. pylori is associated with type B gastritis, duodenal ulcer, gastric ulcer, and gastric cancer. A variety of methods has been developed for diagnosis of H. pylori infection and evaluation of eradication of H. pylori following antibacterial treatment.

U.S. Patent No. 5,498,528 teaches a method for detection of H. pylori strain comprising the steps of contacting a saliva sample suspected of containing H. pylori directly with a urea containing medium selective for growth of H. pylori and

having a pH of about 5.5 to 7.5, and incubating the sample for a time sufficient for detection of H. pylori growth in at least 80% of true positive samples. The method is based on hydrolysis of urea contained in the growth medium by urease enzyme produced by H. pylori and detection of a hydrolysis product by release of a radioactive label from urease or by a color change resulting from action on a pH indicator. The time required to obtain a result by the method disclosed is a function of temperature, approximately 2-3 days when incubated at 23-25ø C and 4-6 hours when incubated at 35-37ø C.

U.S. Patent No. 5,479,935 teaches an ambulatory system for recording and analyzing gastroesophageal reflux. The system comprises a digital recorder, an analysis software package and a catheter for measurement of changes in esophageal impedance. Gastroesophageal reflux can be detected with a pH above 4. The invention allows for recording and analysis of reflux on a non-invasive basis, by using pairs of externally worn impedance sensors.

Other bio-parameters, such as pH or pressure can be measured simultaneously with impedance measurement.

U.S. Patent No. 5,477,854 teaches a system and a method for monitoring intragastrointestinal concentrations of ammonium during prolonged periods, as an indicator of the presence and activity of an intragastrointestinal H. pylori infection. The system may be used in the evaluation of treatments for H. pylori infection in the patient.

U.S. Patent No. 5,439,801 teaches an improved test for the detection of the presence of urease associated with H. pylori in a biopsy specimen. The hydrolysis of lurea by urease is detected by a combination of at least two dye indicators showing a color change. Most positive results occur in 2-10 minutes and all occur in no more than 4 hours.

U.S. Patent No. 5,438,985 teaches a method and a system for ambulatory recording of the pH and the presence of various materials in compartments of the gastro-intestinal tract. The invention also reports the pH pattern in relation to the prevalence of the materials, and analyses to which degree such materials are in active or inactive states in their normal or foreign compartments. This is useful in situations, for example, when duodenal material is refluxed into the stomach and esophagus. The invention involves a gastro-intestinal catheter with a pH sensor and a combined light absorption and fluorescent sensor, a signal recorder and processor, and a written report producer.

U.S. Patent No. 5,420,016 and U.S. Patent No. 5,314,804 teach a rapid method for determining the presence of H. pylori in a biological tissue specimen by detecting the presence of urease in the tissue. The system employs a multilayer test device for the detection of ammonia generated from urea at the presence of urease in the specimen.

U.S. Patent No. 5,420.014 teaches a method of detecting contemporary infection by H. pylori in a mammal. The method is based on the formation of complex between an specific IgG antibody in said mucous secretion and an antigen component from H. pylori. The antigen component is immobilized onto a solid support.

U.S. Patent No. 5,262,156 teaches an assay for detecting H. pylori. The assay involves an ELISA for urine samples, and includes a kit wherein the antigenic composition is immobilized on a solid support.

U.S. Patent No. 4,947,861 teaches a non-invasive method for detection of C. pylori. A breath sample is collected from a patient ten minutes after the patient ingests a quantity of urea. The sample is dehydrated by passing through a solid-state body of alkaline hygroscopic material and analyzed for

ammonia. The presence of ammonia indicates presence of C. pylori in the stomach.

U.S. Patent No. 4,882,271 teaches a method for the serological detection of C. pylori. An antigen for the detection of C. pylori infection is purified from C. pylori. The antigen can be used in a variety of assays including radioimmunoassay, ELISA, latex agglutination, complement fixation, and indirect hemagglutination.

The urea breath test based on the extremely high endogenous urea activity of H. pylori is reliable and non-invasive method with high sensitivity and specificity suitable for diagnosis and evaluation tests; however, the application of the urea breath test is restricted by high cost in isotope labeled material, time, expensive equipment, and undesirable radioactive exposure to C14.

Against this background there remains a need for a method of diagnosing C. pylori that is rapid, non-invasive, and able to distinguish infection in an active state from dormant or non-living residues.

Disclosure of Invention

In accordance with the parent application of which this application is a continuation-in-part, it has been found that a novel non-invasive method for diagnosis of active Helicobacter pylori infection is based on simultaneous detection of antibody to the H. pylori and abnormal level of an ammonia constituent in a body fluid such as blood or saliva. Since antibody to the H. pylori lasts for a long period of time even with eradication of H. pylori, a serological test alone cannot predicate whether the bacteria infection is active. Ammonia concentration in a body fluid is affected by many diseases, and hence ammonia estimation alone cannot pinpoint exact cause of abnormal ammonia value.

Combining the information obtained for ammonia analysis and the serological test, however, affords a novel test for active H. pylori requiring only small samples of body fluid and minimal inconvenience to the patient.

Also in accordance with said parent, a diagnostic apparatus for estimating an active Helicobacter pylori infectious agent in saliva comprises in combination an immunoassay chamber in which a first portion of said saliva is subjected to serological test for antibody to said infectious agent and a chemical reaction chamber in which a second portion of said saliva is subjected to chemical analysis for an ammonia constituent thereof. The presence of antibody to the H. pylori as shown by serological test coupled with abnormal level of ammonia indicates active H. pylori infection, while a positive serological result with normal ammonia constituent level indicates an H. pylori infection that is inactive or limited.

Moreover in accordance with said parent, quantitative or semi-quantitative analysis of ammonia and antibody levels in saliva can be used to monitor the eradication of H. pylori.

A method in accordance with said parent of estimating an active H. pylori biological infectious agent in a body fluid such as blood or saliva, comprises in combination subjecting a first portion of said fluid to serological test for antibody to said H. pylori infectious agent and subjecting a second portion of said fluid to chemical analysis for an ammonia constituent thereof.

The diagnostic apparatus of this invention can be constructed as a single device with two reaction chambers, one for chemical analysis and one for serological test or immunoassay.

The two reaction chambers can also be in physically separate devices used to process for chemical analysis and for serological test portions of a single specimen of body fluid.

In accordance with this invention, an ammonia test device can be incorporated as an integrated part of the diagnostic apparatus or used separately for chemical analysis. The ammonia test device comprises at least one sample layer, a reagent layer impregnated with the necessary reagents for displacing ammonia from the sample, a gas permeable layer for separation of displaced ammonia from sample and reagent, and a sensor layer for detection and estimation of ammonia. The sample layer, reagent layer, gas permeable layer, and sensor layer need not be physically in contact with one another.

In the method of the present invention, a sample of the body fluid to be examined is applied to each of the reaction chambers. Ammonia in the fluid is detected in one chamber with the ammonia test device of the invention. In another chamber, immunochemical information is obtained with immobilized antigen. The immunoassay is based on the formation of antigen-antibody complexes to detect the presence of antibodies or antigens, using antigen to detect antibodies and antibody to detect antigens as required.

Generally, antigens of the infectious agent such as H. pylori are coated on a solid support. Antibodies present in the sample being examined and specific to the antigen are captured on the solid support, resulting in the formation of antigen-antibody complex. A second antibody labeled with radioactive, enzyme, fluorescent, chemiluminescent or other compound with detectable chemical or physical properties is used to detect the presence of antigen-antibody complex through the formation of an antigen - antibody - anti-antibody complex. Thus, if antibody is present in the sample of a fluid such as saliva, antigen-antibody complexes are formed after a period of incubation when the fluid sample is applied

to the chamber. After washing the unbound fluid from the membrane with buffer solution to eliminate non-specific binding, protein-A gold conjugate is added to detect the presence of H. pylori antibodies. The Protein-A Gold conjugate binds to the Fc portion of H. pylori antibodies captured by antigens on the support. A positive reaction for H. pylori antibodies is confirmed by a visible red or slightly pink spot in the test area.

Other features which are considered as characteristic for the invention are set forth in the appended claims.

Although the invention is illustrated and described herein as embodied in estimation of active infection by Helicobacter Pylori, it is nevertheless not intended to be limited to the details shown, since various modifications and structural changes may be made therein without departing from the spirit of the invention and within the scope and range of equivalents of the claims.

The construction and method of operation of the invention, however, together with additional objects and advantages thereof will be best understood from the following description of specific embodiments when read in connection with the accompanying drawings.

Brief Description of Drawings

Fig. 1 is a perspective view of a double-chamber reaction device for carrying out chemical and immunochemical analysis simultaneously according to the invention, in which the chambers are identified by the numbers 1 and 2.

Fig. 2a is a cross-sectional view of the device of Fig. 1 without reagents in the chamber for chemical analysis, 3, and the chamber for immunoassay fitted with reaction membrane 4, absorbance material 5, and cylindrical sample reservoir 6.

Fig. 2b is a cross-sectional view of the device with immobilized reagents 3a at the bottom of the cell for chemical analysis.

Fig. 3a is a perspective view of an ammonia test strip with multi-layer structure to perform reaction, separation, and detection in one step.

Fig. 3b is a cross-sectional view of an ammonia test strip of Fig. 3a.

Fig. 4a is a perspective view of an ammonia test strip capable of performing two individual tests at the same time.

Fig. 4b is a cross-sectional view of an ammonia test strip of Fig. 4a.

Fig. 5a is a perspective view of an ammonia test device including reaction cell 18 and sensor cap 19.

Fig. 5b is an exploded perspective view of top portion of an ammonia test device of Fig. 5a.

Fig. 6 is a cross-sectional view of an ammonia test device which includes a sample layer 33, a reagent layer 34, a gas-permeable layer 35, and a sensor layer 36.

Fig. 7 is a perspective view of an ammonia test device which includes a reaction cell with sample layer and reagent layer disposed on the bottom thereof and sensor layer in the form of a cover slide, with open space inside the reaction cell serving as gas-permeable layer.

Fig. 8a is a perspective view of the reaction cell of the ammonia test device of Fig. 7

Fig. 8b is a top view of the reaction cell of Fig. 8a.

Fig. 8c is a cross-sectional view of the reaction cell along line a-a' of Fig. 8b.

Fig. 8d is a cross-sectional view of the reaction cell along line b-b' of Fig. 8b.

Fig. 9 is an exploded perspective view of the sensor slide of Fig. 7.

Fig. 10 is an exploded perspective view of an ammonia test device which includes a top layer, a backing layer, a sample layer, a reagent layer, a gas-permeable layer, and a sensor layer.

It should be noted that the sketches are not drawn to scale. All the drawings are for illustration of the present invention without limitation..

Best Mode for Carrying Out the Invention

Throughout the present specification and claims, "ammonia constituent" is used to refer to any one or more of the species ammonia gas, ammonium ion, and ammonium hydroxide. It has been found that elevated levels of ammonia constituent can be detected in body fluids of persons with active H. pylori infection, so that detection and estimation of such levels of ammonia constituent, in combination with serological test for antibody to H. pylori, can serve as a diagnostic method for active H. pylori infection.

The diagnostic apparatus according to this invention is suitably characterized as having at least one chamber comprising a hollow container with an opening at one end, as illustrated in Fig. 2a and 2b, Fig. 7, and Fig. 10.

When the container with an open end is the immunoassay chamber, it is suitably characterized as comprising a reaction membrane with immobilized antigen or antibody to said infectious agent, an absorbance layer, and a sample reservoir. The reaction membrane, illustrated at 4 in Fig. 2a, can be any material, organic or inorganic, with sufficient porosity to allow access by samples to be analyzed and with suitable surface affinity to bind antigens.

Useful membrane materials include nylon, glass fiber, and natural or synthetic polymers including cellulose esters. The porosity of the membrane can vary from 0.2 to 12 microns.

A nitrocellulose (i.e. cellulose nitrate) membrane has excellent absorption and adsorption qualities, and is preferred. Mechanical strength of nitrocellulose membrane is greatly improved with a paper or polyester support. Commercially available paper backed nitrocellulose membrane is conveniently used for easy handling. For the relatively high viscosity of saliva a relatively large porosity grade of membrane is a preferred choice. A binding reagent specific to H. pylori antibody, such as a commercially available preparation of H. pylori antigen, is immobilized on the membrane and reacts with and captures H. pylori antibody when present in the sample of fluid. The thickness of the membrane should be sufficient to immobilize a sufficient amount of antigen to provide adequate sensitivity, but not too thick to block the passage of saliva samples.

The absorbance layer, illustrated at 5 in Fig. 2a, serves to draw liquid through the reaction membrane and can be made of any kind of porous hydrophilic absorbent material, suitably filter paper. The sample reservoir, illustrated at 6 in Fig. 2a, is used to apply a sample to the reaction membrane and keep solution running through the center of the reaction membrane containing the immobilized antigen or antibody.

When the container with an open end is the chemical reaction chamber, it is suitably characterized as comprising a hollow reaction chamber as illustrated at 3 in Fig. 2a in which chemical analysis can be carried out in any of several ways. A sample such as saliva can be added to the cell and mixed with all necessary reagents for producing a detectable response to an analyte of interest in 3. Reaction is allowed to proceed for a certain period of time, then the product is separated and analyzed by HPLC, IC, ion selective electrode, uv-visible spectrophotometric method or other analytical method specific to the analyte of interest.

In a particularly preferred embodiment of this invention, the analyte of interest is an ammonia constituent and the test device affording a detectable response to ammonia can be used for detection and estimation thereof. Such a device including reaction cell 27 and sensor slide 28 can, for example, detect ammonia gas generated from an ammonia constituent of a saliva sample placed in the cell.

As illustrated at 3a in Fig. 2b and 34 in Fig. 8c, reagents necessary for chemical analysis can also be pre-mixed and added to the bottom of the cell. 3a can represent a pure single reagent, a mixture of several reagents, or a reagent impregnated strip prepared by drying porous hydrophilic material such as filter paper soaked with reagent solutions. In the case of reagent impregnated strip, it can be conveniently attached to the bottom of 3 using small amount of epoxy or other adhesive material which does not interfere with the reaction. With reagent or filter paper impregnated with all necessary reagents added to the cell first, one step reaction is carried out by adding saliva or other body fluid sample directly to the cell 3.

A detectable response to ammonia in accordance with this invention is a change in color of a pH indicator resulting from the reaction of ammonia generated from a sample being examined with the indicator suitably impregnated on a test strip, sensor layer or sensor slide.

Suitable pH indicators are characterized by a visible color change at a pH in the range from 4 to 12 and include the following which are preferred.

Indicator	pH Range	Color Change
2-(2,4-Dinitrophenylazo)-1-naphthol-3, 6-disulfonic acid	6.0-7.0	yellow-blue
4,4'-bis(4-amino-1-naphthylazo)-2, 2'-stilbenedisulfonic acid	8.0-9.0	blue-red
6,8-dinitro-2,4-(1H)qunizaolinedione	6.4-8.0	colorless-yellow

alizarin	5.6-7.2	yellow-red
brilliant yellow	6.6-7.8	yellow-red
bromothymol blue	6.0-7.6	yellow-blue
cresol red	7.0-8.8	yellow-red
m-nitrophenol	6.8-8.6	colorless-yellow
metacresol purple	7.4-9.0	yellow-purple
neutral red	6.8-8.0	red-amber
phenol red	6.6-8.0	yellow-red
phenolphthalein	8.2-10.0	colorless-pink
rosolic acid	5.0-6.8	yellow-red
thymol blue	8.0-9.6	red-blue
turmeric	7.4-8.6	yellow-red
xylenol blue	8.0-9.8	yellow-violet

Another detectable response to ammonia in accordance with this invention is the formation of highly conjugated indophenol dye absorbing strongly at 630-720 nm by reaction of ammonia and a phenol under oxidizing conditions in the so-called Berthelot reaction (see for example P. L. Searle, Analyst 1984, Vol. 109, pages 549-568). An ammonia test strip based on the Berthelot reaction according to this invention is constructed, for example, by soaking porous hydrophilic material such as filter paper with a strong alkali such assodium hydroxide, an alkali metal salicylate such as lithium salicylate, potassium salicylate, or sodium salicylate, and sodium nitroprusside catalyst, and drying the soaked material so as to avoid overheating, as in an incubator.

A further detectable response to ammonia in accordance with this invention is the formation of a strongly fluorescent product from ammonia and a fluorescence generator reagent such as 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (see for example R. Kobayashi et al, Chem. Pharm. Bull. 1992, vol. 40, pages 1327-28).

Referring to Fig. 3a and Fig. 3b, a one step detection of an ammonia constituent in saliva is achieved by using a multi-layer ammonia test strip according to this invention. Such a test strip can consist of reagent layer 12, gas-permeable layer 13, and sensor layer 14 prepared from porous material. 8 and 9 are supporting layers serving to keep 12, 13, and 14 in position. Paper, such as photocopy paper, is a preferred low cost supporting layer, however synthetic polymers such as polyvinyl chloride can also be used as supporting layer.

Paper glue can be used to stick 8 and 9 together. Reagent layer 12 is impregnated with the necessary reagent for chemical reaction, which for ammonia constituent analysis is a strongly basic compound such as sodium hydroxide or potassium hydroxide.

Gas-permeable layer 13 serves to separate volatile analyte such as ammonia from non-volatile interfering substances such as basic inorganic hydroxides, keeping the latter from reaching sensor layer 14. For the fabrication of gas permeable layer 13, a gas-permeable membrane such as polytetrafluoroethylene, polypropylene, or polyethylene can be used. A particularly preferred gas-permeable layer can be conveniently prepared using cellulose acetate butyrate coated filter paper. Alternatively, the gas-permeable layer is simply an unfilled space through which ammonia gas is free to rise and contact sensor strip, sensor layer, or sensor slide while gravity prevents liquids and solids from rising.

Sensor strip 14 is prepared with pH indicator as illustrated above or with reagents for Berthelot reaction or with fluorescence generator.

When a sample such as saliva is applied to the reagent layer through the hole 10, ammonia constituent in the sample reacts with strong base such as sodium hydroxide in reagent layer and releases ammonia gas. Ammonia gas diffuses through gas-permeable layer 13 and a distinct color change or

fluorescence is observed through hole 11 when ammonia reaches sensor layer 14.

In a particularly preferred embodiment, there can be on the ammonia test strip a plurality of sensing units each having an assembly of layers 12, 13, and 14 as shown in Fig. 3b. In this way, two or more samples can be analyzed at the same time using a single test strip as shown in Fig. 4a and Fig. 4b. Semi-quantitative information can be obtained by running standard and sample simultaneously. To avoid interference, there should be enough space, suitably 8 millimeters, between two sample wells 15 and 16. A single large piece of hydrophobic gas-permeable layer 17 (Fig. 4b) can be used to avoid diffusion of solution from one cell to another.

Referring to Fig. 5a and Fig. 5b, a saliva analyzer for a volatile organic or inorganic analyte such as an ammonia constituent in accordance with this invention includes a reaction cell 18 and a detection cap 19. The reaction cell 18 can be made of any material resistant to chemical attack during the chemical analysis, suitably of glass or plastic. The reaction cell 18 has an open end. The cap 19 can be threaded or clipped onto the cell 18. There is a hole 20 in cap 19, suitably in the middle thereof. A chemical sensor strip 21 can be fixed onto the cap through a solid backing layer 22 with a hole aligned with 20.

Backing layer 22 when used is made of plastic or paper with adhesive material on one side. Instead of using backing layer 22, the sensor strip can be glued directly to the base of the cap using epoxy or other adhesive material.

The sensor strip 21 responds to volatile product, such as ammonia, from the reaction inside the cell 18, resulting in color change, physical property change such as resistance, or the formation of a product which can be determined by various analytical methods such as GC or HPLC. When a sensor strip is

impregnated with a pH indicator such as phenol red or cresol red, a distinct color change can be observed through the hole 23 in the backing layer 22.

The sensor strip can also be prepared right before the test by applying indicator solution to the strip through top opening 23.

Referring to Fig. 6, an ammonia test device according to this invention is suitably characterized as having a hollow tube filled with different layers with different functions. The tube 31 can be any shape with empty space inside. It can be made from any kind of material which is insoluble in contact with water, saliva or blood etc. The tube 31 can be made of glass, plastic, steel, copper, or aluminum etc. The wall 32 should be stiff with certain mechanical strength in order to pack the inside of the tube with sample layer 33, reagent layer 34, gas-permeable layer 35, and sensor layer 36. Glass or plastic tubing with transparent wall 2 is a preferred choice. Sample layer 33 is made of porous material. It is used to carry liquid sample to the reaction layer 34. Reagent layer 34 is porous material pre-soaked with sodium hydroxide, potassium hydroxide or other strongly basic material. Gas-permeable layer 35 is used to prevent liquid to reach sensor layer 36. Sensor layer 36 consists of porous material soaked with pH indicator such as phenol red etc.

When the test device is in contact with saliva, blood or other body fluids in a container, samples can wet the sample layer 33 and be carried toward the reagent layer 34. Ammonia constituent in the sample will react with sodium hydroxide in the reagent layer 34, releasing ammonia gas. Ammonia gas goes through gas-permeable layer 35 to reach sensor layer 36, resulting in distinct visible color change. Since liquid cannot go through gas-permeable layer 25, interference from sodium hydroxide can be effectively eliminated.

Referring to Fig. 7 in conjunction with Fig. 8a - Fig. 8d and FIG. 9, the ammonia test device includes a reaction cell 37 and a sensor slide 38. There is a hole 39 in the sensor slide for observation of color change following chemical reaction in the reaction cell.

Polymers or metals can be used to prepare the reaction cell and the sensor slide. The reaction cell 40 consists of a hollow container with one open end 41 and slots 42. Samples can be added to the reaction cell through the opening 43. Reagents 44 necessary for chemical reaction can be deposited on the bottom of the reaction cell. Reagent 44 can be a single chemical such as pure sodium hydroxide, a mixture of different chemicals, or a porous material soaked with chemicals. There are two purposes for the slots 45 (Fig. 8d) in the reaction cell, first, to hold the reaction cell 37 and the sensor slide 38 tegether, and second, to position the opening of the sensor slide 37 right on the top of the opening 43 of the reaction cell 41. The sensor slide 46 is attached to the reaction cell through the base 48. Ammonia gas released from the reaction cell can go upward through the opening 43 of the reaction cell and opening 47 of the sensor slide to reach the sensor layer 49. Visible color change can be observed through a hole 51 in the backing layer 50. The sensor layer contains pH indicator, suitably impregnated on porous material. The backing layer is used to attach the sensor layer 49 to the sensor slide 46. There is adhesive material on the bottom of 50 and the size of 50 is larger than that of 49. The sensor layer 49 can be easily attached to the slide 46 by pressing 50 against 46. Referring to Fig. 10, the ammonia analysis device includes a backing layer 52, a top layer 53, a sample layer 56, a reagent layer 57, a gas-permeable layer 58, and a sensor layer 59. A piece of filter paper or other porous material is used to prepare layers 56, 57, and 59. The reagent layer 57 is prepared by soaking and

drying a small piece of filter paper with sodium hydroxide solution. The sensor layer is prepared by soaking and drying a piece of filter paper with phenol red or other pH indicator solutions. Gas-permeable layer can be made from polymeric gas-permeable membrane such as tetrafluoroethylene polymer. Layers 56-59 are secured to the backing layer 52 by gluing or heat-sealing top layer to the backing layer 52. There are two openings 54 and 55 in the top layer 53. The opening 54 is used to add sample to the sample layer 56. The opening 55 is used to observe color change following chemical reaction. Sample is added to porous sample layer 56 through window 54. Sample is carried to the reagent 57. An ammonia constituent such as ammonium ion will react with sodium hydroxide in the reagent layer 57 and resulting ammonia gas goes through gas-permeable layer 58 and reaches the sensor layer 59 resulting in a distinct color change.

Further disclosure of the invention is provided by the following examples, offered for purpose of illustration and not of limitation.

Example 1 - Simultaneous chemical and immunochemical analysis In this instance, a device of the type of Fig. 1 was used to carry out chemical and immunochemical analysis.

100 microliters of 10 mM ammonium chloride was added to the cell 1. Then one drop of 2 M sodium hydroxide was added to the cell. When a test strip impregnated with phenol red indicator was placed on top of the opening, instant color change was observed, indicating the release of ammonia from the reaction.

As an example of immunoassay, H. pylori antigen was immobilized on the reaction membrane 4 in Fig. 2a. Serum or saliva sample containing antibodies to H. Pylori was tested using the chamber 2 in Fig. 1. After the sample was absorbed totally through the reaction membrane 4 by absorbence layer 5

in Fig. 2a, washing reagent phosphate buffer containing Tween 20 surfactant and heat treated normal goat serum was applied. When the washing solution was absorbed, protein A - gold conjugate was applied. The treated reaction membrane was incubated at room temperature for 10 minutes and washed again with the washing reagent. A red dot in the middle of the reaction membrane indicated the presence of H. pylori antibodies in the sample.

Example 2 - Ammonia test strips

In this example, preparation of ammonia test strips based on the Berthelot reaction is described. 4.08 g sodium hydroxide (Sigma Chemical Company), 1.19 g sodium salicylate (Aldrich), and 0.05 g sodium nitroprusside (Sigma) were ground into small particles in a porcelain mortar using a porcelain pestle. 5 ml water was added and grinding continued until a fine solid suspension was obtained. The mixture was spread over a piece of Whatman #4 qualitative filter paper and the paper soaked with the reagent was dried overnight in a 40ø C incubator. The filter paper treated in this way was used as an ammonia test strip.

The performance of the test strip was tested using 10 mM ammonium chloride solution. 100 microliters 10 mM ammonium chloride solution was mixed with 100 microliters 5% sodium hypochlorite solution (Sigma). The mixture was applied to the test strip. The color of the strip changed from yellow to green within five minutes.

Example 3 - Ammonia test strips with gas-permeable layer

Ammonia test strip shown in Fig. 3a was fabricated. Sensor layer was prepared from 0.02% phenol red or credsol red (Sigma) by soaking and drying a Whatman #4 qualitative filter paper. Gas-permeable layer was prepared by coating a piece of Whatman #4 filter paper with cellulose acetate butyrate.

Reagent layer was prepared from 2 M sodium hydroxide solution. All strips were cut into 1 cm x 1cm square pieces. As shown in Fig. 3b, a reagent layer piece, as gas-permeable layer piece, and a sensor layer piece were stacked glued together using two pieces of paper with one hole at one side of the end.

The performance of the test strip was tested using 2 M sodium hydroxide and 10 mM ammonium solution. When one drop of sodium hydroxide solution was applied to hole 10 in Fig. 3b, no color change was observed through hole 11 in Fig. 3b, indicating no leakage of solution through the gas-permeable layer. When ammonium solution was applied, almost instant color change was observed resulting from pH change in the sensor strip due to dissolved ammonia. Saliva sample was also analyzed with the test strip. The test strip was sensitive enough to detect ammonia constituent in the saliva. The ammonia in the saliva was in the millimolar range.

Ammonia test strip as shown in Fig. 4a was prepared using similar procedure. 2.0 mM, 4.0 mM, 6.0mM, and 8.0 mM ammonium chloride solutions were used to evaluate the performance of the test strip. Two samples were analyzed simultaneously. There was distinct difference in color intensity and color development time when concentration changed with 2.0 mM increment, indicating the possibility to use such a kind of test strip to get semi-quantitative information.

In order to increase color contrast and increase ammonia collection efficiency, test strips are wetted using small volume of distilled water right before analysis.

Claims

1. An ammonia test device, comprising a sample layer, a reagent layer, a gas-permeable layer, and a sensor layer.

2. An ammonia test device according to claim 1, in which a hollow tube is filled at least with one reagent layer and one sensor layer.

3. An ammonia test device according to claim 1 in which a sample layer comprises porous material such as filter paper.

4. An ammonia test device according to claim 1 in which a reagent layer is porous material soaked with sodium hydroxide or potassium hydroxide.

5. An ammonia test device according to claim 1 in which a gas-permeable layer comprises gas-permeable membrane such as tetrafluoroethylene polymer.

6. An ammonia test device according to claim 1 in which a sensor layer comprises pH indicators.

7. An ammonia test device according to claim 6 in which said pH indicator is selected from the group consisting of 2-(2,4-Dinitrophenylazo)-1-naphthol-3,6-disulfonic acid, 4,4'-bis (4-amino-1naphthylazo)-2,2'-stilbenedisulfonic acid, 6,8-dinitro-2,4-(1H)qunizaolinedione, alizarin, brilliant yellow, bromothymol blue, cresol red, m-nitrophenol, metacresol purple, neutral red, phenol red, phenolphthalein, rosolic acid, thymol blue, turmeric, and xylenol blue.

8. An ammonia test device comprising a reaction cell and a sensor slide.

9. An ammonia test device according to claim 8 in which a reaction cell comprises a reaction chamber and slots.

10. An ammonia test device according to claim 9 in which a reaction chamber comprises a porous material soaked with sodium hydroxide or potassium hydroxide.

11. An ammonia test device according to claim 8 in which a sensor slide comprises a sensor strip and a backing layer.

12. An ammonia test device according to claim 11 in which a sensor strip comprises pH indicators listed in claim 7.

13. An ammonia test device comprising a backing layer, a support layer, a sample layer, a reagent layer, a gas-permeable layer, and a sensor layer.

14. An ammonia test device according to claim 13 in which a support layer, a reagent layer, and a sensor layer comprises porous material such as filter paper.

15. An ammonia test device according to claim 13 in which a reagent layer comprises filter paper soaked with sodium hydroxide or potassium hydroxide.

16. An ammonia test device according to claim 13 in which a sensor layer comprises filter paper soaked with pH indicators listed in claim 7.

17. An ammonia test device according to claim 13 in which said a gas-permeable layer comprises gas-permeable membrane such as tetrafluoroethylene polymer.

18. An ammonia test device according to claim 13, in which a backing layer and a support layer comprises polymers such as polyethylene, propylene, and polystyrene or inorganic materials such as aluminum, steel, and copper.

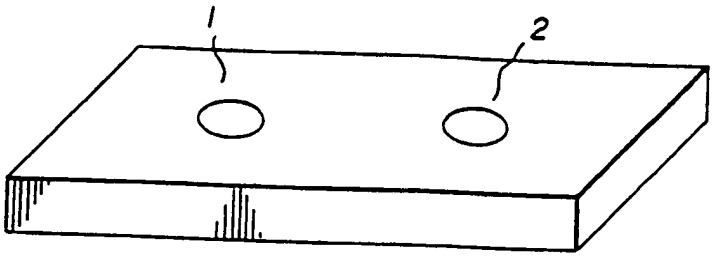

FIG. 1

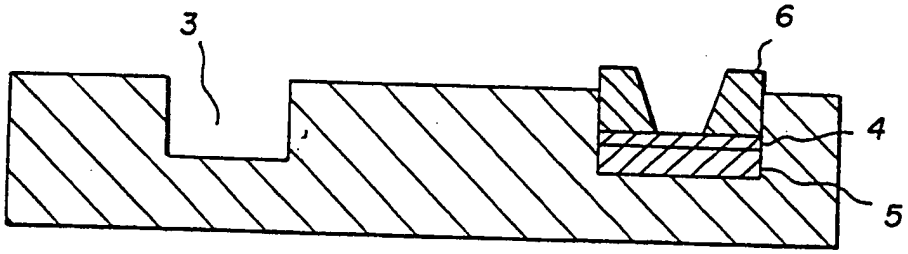

FIG. 2A

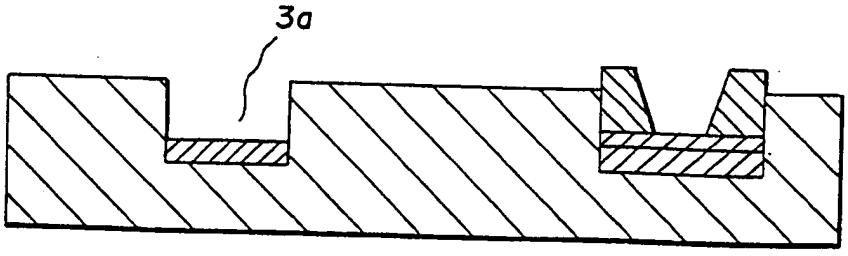

FIG. 2B

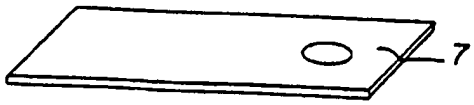

FIG. 3A

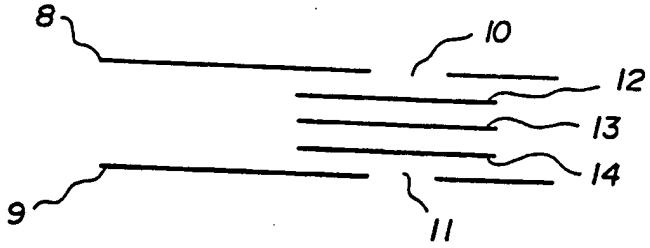

FIG. 3B

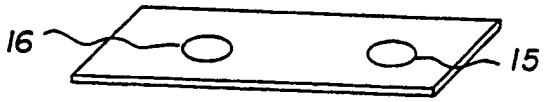

FIG. 4A

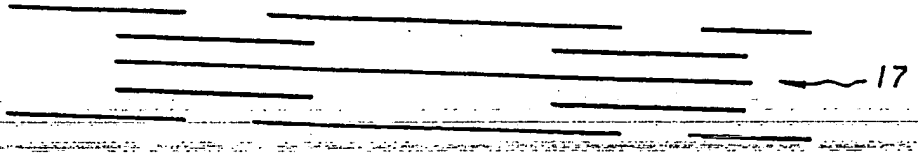

FIG. 4B

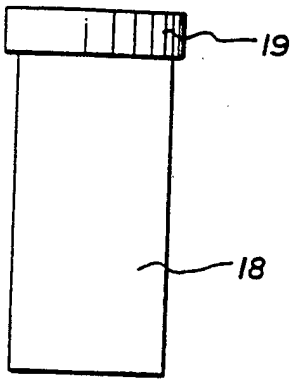

FIG. 5A

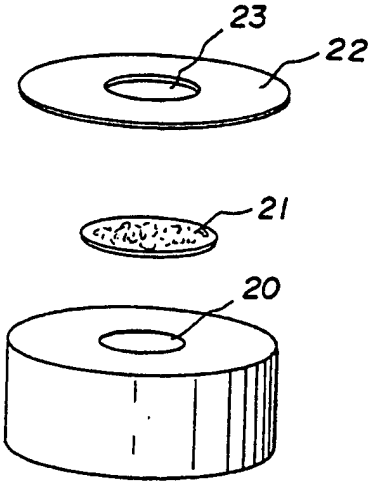

FIG. 5B

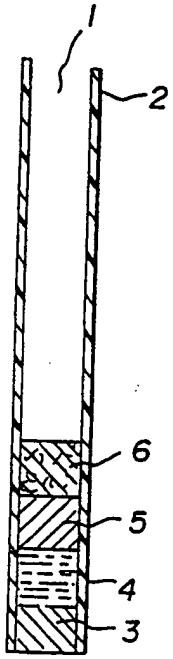

FIG. 6

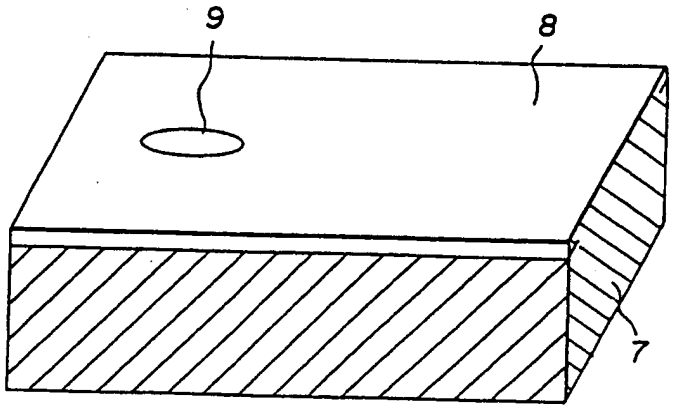

FIG. 7

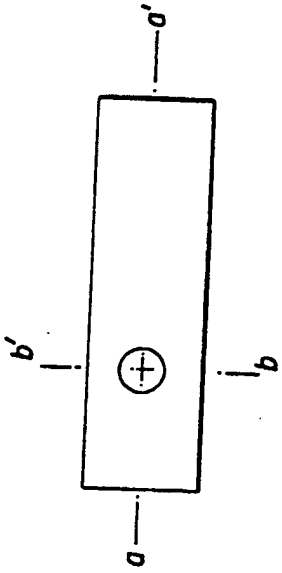

FIG. 8B

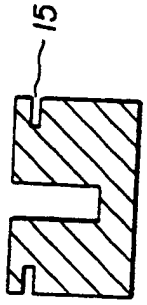

FIG. 8D

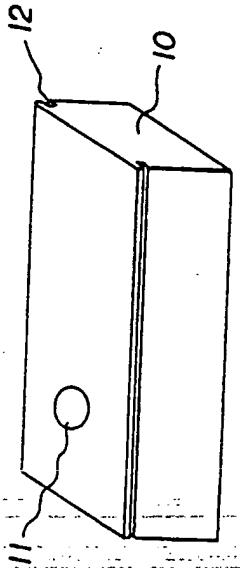

FIG. 8A

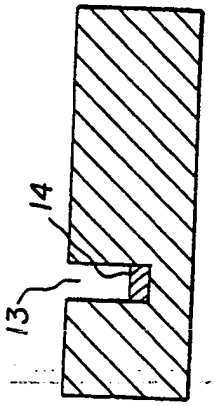

FIG. 8C

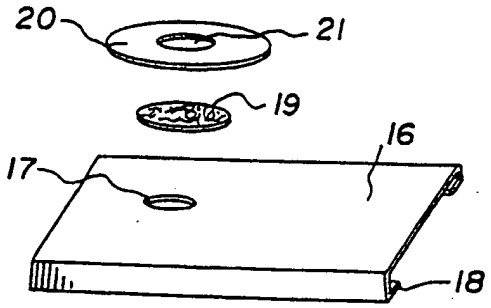

FIG. 9

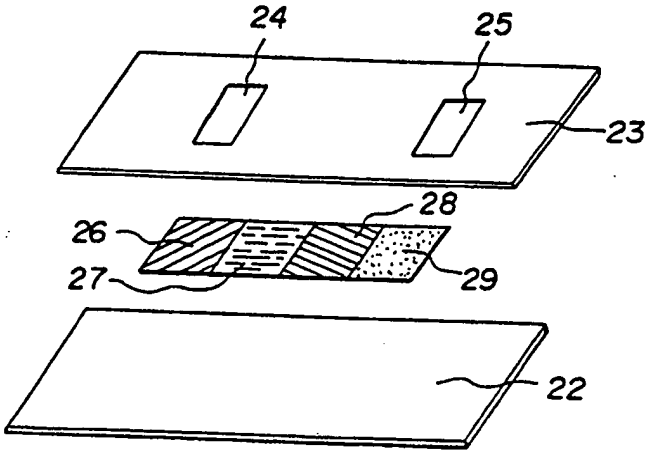

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05441

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 21/00, 31/22

US CL : 422/40, 68.1,56,57,98; 435/12,98; 436/111,113; 204/403

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/40, 68.1,56,57,98; 435/12,98; 436/111,113; 204/403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,554,339 A (COZZETTE et al) 10 September 1996, see figure 7a and entire document.	1,3-6,8-13,15,17N
X,P	US 5,709,837 A (MORI et al) 20 January 1998, see abstract, claims 9 and 19, and entire document.	1,3,4,6,7
X	US 5,286,624 A (TERASHIMA et al) 15 February 1994, see abstract, col. 3-5, 8-9, and claims.	13,14,16, 18
X	US 5,420,016 A (BOGUSLASKI et al) 30 May 1995, see abstract and entire document.	1,3,5-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
	20 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05441

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,066,403 A (BRUSCHI) 03 January 1978, see figures, abstract, col. 3-4, and 5 lines 55-64, col. 7, lines 9-17 and lines 40-45, col. 10, lines 14-18, col. 12-17 and entire document.	1,3,6,7,13, 16,18
X	US 4,769,216 A (CHANDLER et al) 06 September 1988, see abstract, figures, col. 2, 6-8 and entire document.	2
X	US 4,426,451 A (COLUMBUS) 17 January 1984, see figures, col. 15-16 and entire document.	1-3, 6-7
X	US 4,297,173 A (HIKUMA et al) 27 October 1981, see abstract, col. 2,4 and entire document.	13,18
X	US 5,252,292 A (HIRATA et al) 12 October 1993, see abstract, col. 3-4 and entire document.	8,11
X	US 4,719,085 A (JACOBS) 12 January 1988, see abstract, figures, col. 1-2 and entire document.	1
X	US 5,344,546 A (KIESELE et al) 06 September 1994, see col. 2-3, 7-10 and entire document.	8,11,13
X	US 4,350,660 A (ROBINSON et al) 21 September 1982, see entire document.	8-9,11
X	US 5,198,335 A (SEKIKAWA et al) 30 March 1993, see col. 5-6 and entire document.	1,3,6-7,13-14, 17-18
X	US 4,548,906 A (SEKIKAWA et al) 22 October 1985, see abstract, col. 3-4 and entire document.	1,3,6-7,13,14,16
X	US 5,091,080 A (van EIKEREN et al) 25 February 1992, see abstract, col. 2-3, and entire document.	1,3,5
X	US 5,008,078 A (YAGINUMA et al) 16 April 1991, see abstract, col. 2-6 and entire document.	1,3-4,6-7
Y	US 5,238,613 A (ANDERSON) 24 August 1995, see abstract, col. 24, line 30 and entire document.	8,11
Y	US 5,443,080 (D'ANGELO et al) 22 August 1995, see abstract, figures and entire document.	1,3,6,13,14
Y	US 5,411,893 A (EDEN et al) 02 May 1995, see abstract, figures, col. 3-6 and entire document.	1,3,6,7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05441

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,116,759 A (KLAINER et al) 26 May 1992, see abstract, figures, claims and entire document.	8,9,11,12
X	KOBAYASHI, R. et al. Gas phase derivatization of ammonia with 4-fluoro-7-nitrobenzo-2axa-1,3-diazole and its application to urease assay. Chemical and Pharmacological Bulletin, 1992, Vol. 40, No. 2, pages 1327-1328, see entire document.	2
X	HUIZENGA, J.R. et al. Determination of ammonia in saliva using indophenol, an ammonium electrode and an enzymatic method: a comparative investigation. Journal of Clinical Chemistry and Clinical Biochemistry. 08 August 1982, Vol. 20, No. 8, pages 571-574, see entire document.	1
X	WOLFBEIS, O.S. et al. Fibre-optic fluorescing sensor for ammonia. Analytica Chimica Acta, 1986, Vol. 185, pages 321-327, see entire document.	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05441

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05441

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG
search terms: ammonia, ammonium, nh3, ?sensor?, detect?, determin?, apparat?, device?, measur?, method?, ?layer?, urease?, saliva?, salivary?, helicobacter?, pylori

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s)1-7, drawn to an ammonia test device comprising 4 layers.
Group II, claim(s) 8-12, drawn to an ammonia test device comprising a reaction cell and a sensor slide.
Group III, claim(s) 13-18, drawn to an ammonia test device comprising 6 layers.
The inventions listed as Groups I,II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: each group presents a distinct invention as the structural components differ, wherein group I comprises 4 layers of components, Group II, comprises 2 components and Group III comprises 6 components; while each device is used for the detection of ammonia the specific special technical features differ from each other as the number of layers and the type of layers used for the detection of ammonia differ and therefore do not have a linking characteristic; therefore, lack of unity of invention exists.